Br. J. Pharmac. (1981), **72**, 597–602

INACTIVATION OF HERPES SIMPLEX VIRUS BY A PREGNENE DERIVATIVE (TX 3047)

BERNARD PERBAL[1]

Centre de Biochimie, Université de Nice, Parc Valrose, 06034 Nice, France

1 Two strains of independently purified Herpes simplex virus type 1 were irreversibly inactivated after incubation with the pregnene derivative methyl-*p*-toluene sulphonate of diethylamino-3-β-ethoxy-20-hydroxy-pregn-5-ene (TX 3047) *in vitro*.

2 RNA and DNA synthesis (both cellular and viral) were studied in the presence of TX 3047. Viral and cellular DNA synthesis but not viral RNA synthesis was reduced.

3 Formation of infectious particles was not affected by TX 3047 if the parental virus was adsorbed to the cell before it was incubated with TX 3047. This suggests that TX 3047 acts at the level of the membrane.

Introduction

Since the discovery, more than 10 years ago, that 5-iodo-2-deoxyuridine (IUdR) was able to modify the course of induced herpetic keratitis in rabbits, several other compounds, including nucleoside analogues have been tested in animals or humans. Unfortunately progress in human therapy has been very limited. Its molecular and clinical aspects have been extensively reviewed (Prusoff & Goz, 1973; Jawetz, 1973).

In this study, the viricidal properties of the pregnene derivative methyl-*p*-toluene sulphonate of diethylamino-3-β-ethoxy-20-hydroxy-pregn-5-ene (TX 3047) (Figure 1) have been tested *in vitro*.

Methods

Cell cultures and virus strains

The following cell types were used: baby hamster kidney (BHK 21 C13) from Dr J.L. Montagnier (Paris); HeLa from Dr R. Cassingena (Paris); CV1 and Hep2 from Dr J.H. Subak-Sharpe (Glasgow); and FR 3T3 rat cells isolated in our laboratory by Seif & Cuzin (1977). Cells were grown in Dulbecco modified Eagle's medium (GIBO) supplemented with 10% newborn calf serum (GIBCO) and 10% tryptose phosphate (DIFCO).

Herpes simplex virus (HSV) type 1 17 mp was kindly provided by Dr J.H. Subak-Sharpe, and strain P431 was obtained from an isolate taken from a patient in the Centre Hospitalier Regional de Nice by Dr J.P. Cassuto. Both virus stocks were purified by growing stocks from single plaques. BHK 21 cell monolayers were infected with virus dilutions so as to give very few plaques per plate. After 3 days of incubation at 31°C, the culture medium was discarded to remove any extracellular virus which might have appeared during plaque formation, and fresh medium was added to the plates. The virus from single non-overlapping plaques was taken up with finely drawn-out Pasteur pipettes, under a dissecting microscope. The virus was released by sonication for approximately 1 min and used to infect a new set of cells. After incubation for 2 to 3 days at 31°C, the same procedure was repeated. Single plaque purification was successively carried out three times. The resulting purified virus was kept as seed stock.

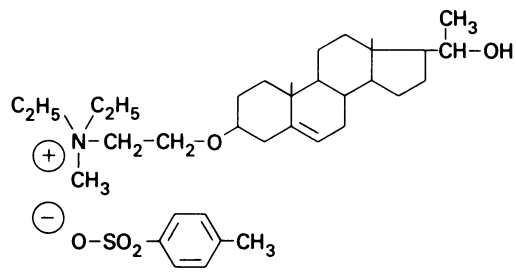


Figure 1 Structure of the methyl-*p*-toluene sulphonate of diethylamino-3-β-ethoxy-hydroxy-pregn-5-ene (TX3047).

[1]Present address: University of California School of Medicine, Department of Pathology, CHS 13–188, Los Angeles, California 90024, U.S.A.

0007–1188/81/040597–006 $01.00

Virus titration

BHK 21 cell monolayers (4×10^6 cells/ 50 mm plate) were infected by logarithmic dilutions of the initial stock. To standardize the experiments, the kinetics of adsorption of the P431 and 17mp strains were estimated in the following way: 0.1 ml of virus dilutions mixed with 0.1 ml of complete medium were added to cell monolayers; at various times, the virus was discarded and 5 ml of complete medium containing 0.5% purified human gamma globulins (Merieux) added to the cells in order to avoid secondary infections; after 18 h of incubation at 37°C the medium was discarded, the cells stained with Giemsa stain (Rhone-Poulenc) and the HSV plaques counted under a Wild Dissecting Microscope. The counts showed that with both strains the majority of the infectious viral particles (plaque forming units: p.f.u.) were adsorbed within 30 min of incubation. An incubation time of 45 min was chosen in all experiments.

TX 3047

TX 3047 was a kind gift from Theramex Laboratory-Monaco. Stock solution (2.5 mg/ml in distilled water) was filtered at 37°C through a 0.22 μm Millipore filter before use. The pH of the solution was 7.2; the solution became a gel when kept at 4°C.

Measurements of viral and cellular mucleic acids synthesis

BHK 21 cell monolayers were infected with HSV-1 17 mp so as to produce 50 p.f.u. per cell. Viral and cellular DNA were labelled by the addition of [^{3}H]-thymidine (100 μCi) to the medium. After labelling, cells were centrifuged at 600 *g* for 10 min and resuspended in 4 ml of 0. 15 M sodium chloride, 0.1 M disodium edetate (EDTA), pH 8.2, 2% sodium dodecyl sulphate (SDS). The suspensions were heated at 60°C for 10 min and then chilled in ice before CsCl (1.29 g/ml) was added. Their density was adjusted to 1.717 with a refractometer (refractive index 1.4015). Viral and cellular DNA (ρ_v = 1.725 g/cm^3 and ρ_c = 1.700 g/cm^3) were then separated by centrifuging for 72 h in 50 Ti rotor (Beckman). Gradients were fractionated from the bottom by pumping. The radioactivity of each fraction was measured by transferring 50 μl aliquots to Whatman No. 1 discs (2.5 cm diameter), which were washed in 10% TCA before drying and counting in standard PPO-POPOP scintillation fluid.

Protein measurements

The colorimetric method of Bramhall, Noack & Loewenberg (1969) as modified by Gaudray (1976) was used. Samples were spotted on Whatman No. 1 discs (2.5 cm diameter), which were dipped in a mixture of G250 Coomassie Brilliant Blue (10 mg/ml in 50% methanol 10% acetic acid) for 15 min at 60°C. Excess dye was removed by washing with hot (60°C) 10% TCA and the filters dried. Each disc was put in a test tube, covered with 3 ml of the destaining solution (66 ml methanol, 34 ml water, 1 ml concentrated ammonia) and the amount of dye released measured with a spectrophotomer at 650 nm. Solutions of bovine serum albumin were used as standard.

RNA-DNA hybridization

Viral RNA was labelled by the addition of [^{3}H]-uridine to the culture medium.

Total labelled cell RNA was purified by phenol/SDS extraction: 10 ml samples in Tris-NaCl-EDTA buffer (0.1 M NaCl, 0.01 M EDTA, 0.05 M Tris-HCl pH 7.5) containing 1% (w/v) SDS were mixed with 10 ml of phenol fully equilibrated with the same buffer. After shaking and centrifugation, the upper phase containing RNA, was pipetted and the extraction repeated. RNA was precipitated at −20°C with 2 vol of ethanol (0.1 M NaCl). Excess DNA hybridization (Jamieson, MacNab, Perbal & Clements, 1976) was performed on nitrocellulose filters (Schleicher & Schull Ltd. type BA85) loaded with HSV purified DNA (5 μg) or with λDNA (5 μg). Hybridizations were carried out in 60% (v/v) formamide, 2 × SSC (standard saline citrate is 0. 15 mM sodium citrate) at 42°C for 20 h, with occasional shaking. Filters loaded with λ DNA were used as controls. After incubation, the filters were washed twice by vortexing in 10 ml of 2 × SSC, and then treated with pancreatic RNAse (25 μg/ml) for 40 min at room temperature. The radioactivity associated with λ DNA (representing non-specific binding) was subtracted from the value obtained with HSV filters, and the amounts of specific HSV RNA expressed as % ct/min which hybridized to purified HSV DNA:

$$\frac{\text{HSV RNA counts}}{\text{total RNA counts}}$$

Results

Inactivation of Herpes simplex virus type 1 by TX 3047

When 0.1 ml samples of the HSV 1 stocks (titres ranging from 1–8 × 10^9 p.f.u.) were incubated in the presence of TX 3047 (250 μg) for 5, 10, 15, 30 or 60 min, no infectious virus could be recovered. With 15 min incubation (Table 1), inactivation occurred only in a narrow range of drug concentration (100 to 150 μg). Inactivation was similar when virus treated with TX 3047 was washed twice by centrifugation (25,000 *g* in a JA 20 Beckman rotor) before titration, indicating

that the observed loss of infectivity was not due to the presence of residual TX 3047.

Some of the plaques that appeared in the presence of high concentrations of TX 3047 were collected and submitted to three successive purifications in the presence or in the absence of TX 3047. None of the stocks grown from the resulting virus produced a resistant mutant strain. No infectious virus could be recovered after extensive washing of the treated virus, with either 5 × 10 ml of culture medium, phosphate buffer (pH 7.5 10^{-2} M) or Tris-HCl buffer (pH 8.0 10^{-2} M), followed by plating aliquots of the washed stocks at intervals of several days on BHK-21 cells. Thus the alteration(s) induced by TX 3047 could not be reversed by washing.

Growth of uninfected cells in the presence of TX 3047

To determine whether TX 3047 interfered with the HSV-1 lytic cycle, plaque formation was followed in cells infected by untreated virus and grown in the presence of TX 3047.

All four cell lines were grown in the presence of increasing concentrations of TX 3047 to test whether a cytotoxic effect could interfere with the production of infectious viral particles by cells infected with HSV-1. Plates were seeded with 5×10^5 cells and the medium changed every 24 h to avoid a possible degradation of the pregnene derivative in the presence of growing cells. Figure 2 shows results with BHK 21 cells. Anchorage of the cells to plastic was not impaired by TX 3047 (up to 100 μg per plate). Concentrations lower than 50 μg/plate caused no detectable change in the cellular morphology or growth rate, either within the time usually required for the appearance of HSV-1 plaques (18 h) or within several days of growth. The growth rate of cells was decreased (doubling time 24 to 40 h) by 50 to 100 μg of TX 3047 per plate. Cytopathic effects were produced by 50 μg of TX 3047 after 62 h of incubation, and by 500 and 750 μg after 24 h. When fresh medium without TX 3047 was added to cells previously grown in the presence of 50 μg TX 3047 for 48 h (before appearance of cytopathic effects), growth rate and cellular morphology were similar to those of cells not exposed to TX 3047.

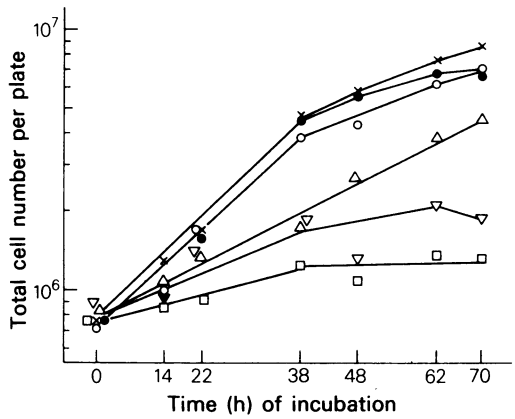


Figure 2 Inhibition of cell growth by TX 3047. BHK 21 cells were grown in the presence of increasing concentrations of TX 3047. Duplicate plates were seeded with 5×10^5 cells. At the indicated times, cells were trypsinized and viable cells counted after addition of trypan blue. Average values from two independent experiments are shown. Concentrations of TX 3047 (μg/ml medium): (X) 0; (●) 10; (○) 25; (△) 50; (▽) 75; (□) 100.

HSV-1 plaque formation in the presence of TX 3047

BHK 21 cell monolayers were infected with HSV 17 mp or P431 and incubated in the presence of 0 to 100 μg of TX 3047 per 1 ml plates. After 18 h incubation at 37°C (no cytopathic effect in control cells; growth rate reduced as in Figure 2), the number of plaques obtained in the presence and in the absence of TX 3047 was the same, indicating that once HSV-1 had been adsorbed on the cells, its lytic cycle was not affected by TX 3047.

Synthesis of viral and cellular nucleic acids in the presence of TX 3047

BHK 21 cell monolayers were infected with HSV-1 17 mp (at an input ratio of 50 viral p.f.u. per cell) and incubated in the presence of 150 μg of TX 3047. Viral DNA and cellular DNA were labelled with [^{3}H-] thymidine 1 to 3 h after infection (early label) or 4 to

Table 1 Inactivation of Herpes simplex virus type-1 (HSV-1) by TX 3047 (15 min incubation at 37°C)

TX 3047 (μg)	0	20	50	100	150
HSV-1 (17 mp) (p.f.u/ml)	3.1×10^8	2.1×10^8	2.5×10^8	1×10^8	3.5×10^3
HSV-1 (431) (p.f.u/ml)	7.2×10^6	6.7×10^6	4.0×10^6	4.4×10^3	0

A volume of virus stocks was mixed with 0.1 ml of TX 3047 and the titre (p.f.u/ml) measured after incubation.

6 h after infection (immediate label); viral and cellular RNA were labelled with [5-^{3}H-]uridine (Jamieson *et al.*, 1976) in duplicate plates incubated under the same conditions. Viral and cellular DNA were purified (Halliburton & Timbury, 1976). The extent of inhibition of cellular DNA synthesis in uninfected cells grown in the presence of TX 3047 (Table 2) was similar to the decrease of cellular growth rate observed under the same conditions (Figure 2). In infected cells, cellular DNA synthesis was decreased by HSV infection (Halliburton & Timbury, 1976) but was less sensitive (39%, 43%) to TX 3047. In contrast viral DNA synthesis was inhibited to the same extent (62%) as that of fully replicating cellular DNA. One set of cells was grown 10 h in the presence of TX 3047 (50 μg) and washed twice with 5 ml of Eagle's medium before infection. Such pretreatment did not interfere with subsequent viral and cellular DNA synthesis (Table 2). TX 3047 significantly reduced cellular RNA synthesis (Table 2) but not HSV-1 RNA synthesis. When cells were first grown in the presence of 50 μg TX 3047 for 10 h and then washed before infection, the synthesis of both viral and cellular RNA was similar to that occurring in control infected cells grown in the absence of TX 3047.

Discussion

The observed irreversible inactivation *in vitro* by TX 3047 of two independently isolated strains of Herpes simplex virus type 1, may be due either to a membrane alteration of the infectious particles (HSV-1 is an enveloped virus) or to a subsequent inhibition of the HSV-1 lytic cycle. Our results suggest that the completion of the viral lytic cycle was not affected by TX 3047.

The present experiments did not determine whether the inhibition of both viral and cellular DNA synthesis was due to a direct effect on DNA replication steps or to an indirect effect such as a modification of intracellular nucleotide pools. Since the amount of viral DNA synthesized in the infected cells is much greater than the total DNA found in the viral progeny (Ben-Porat & Kaplan, 1973) it was not surprising that the 60% inhibition of viral DNA was not accompanied by a decrease in the number of plaques produced by the infected cells. TX 3047 inhibited cellular RNA synthesis but not HSV-1 RNA synthesis. This may indicate that viral RNA synthesis involves a viral coded factor which would not be sensitive to TX 3047 or would render the cell RNA polymerase less sensitive. The involvement of a viral factor in HSV-1 transcription has been suggested (Ben Zeev & Becker, 1977).

As TX 3047 did not affect the course of HSV-1 production once the virus was adsorbed on the cells, the drug may induce loss of infectivity by modifying the membrane of the treated virus.

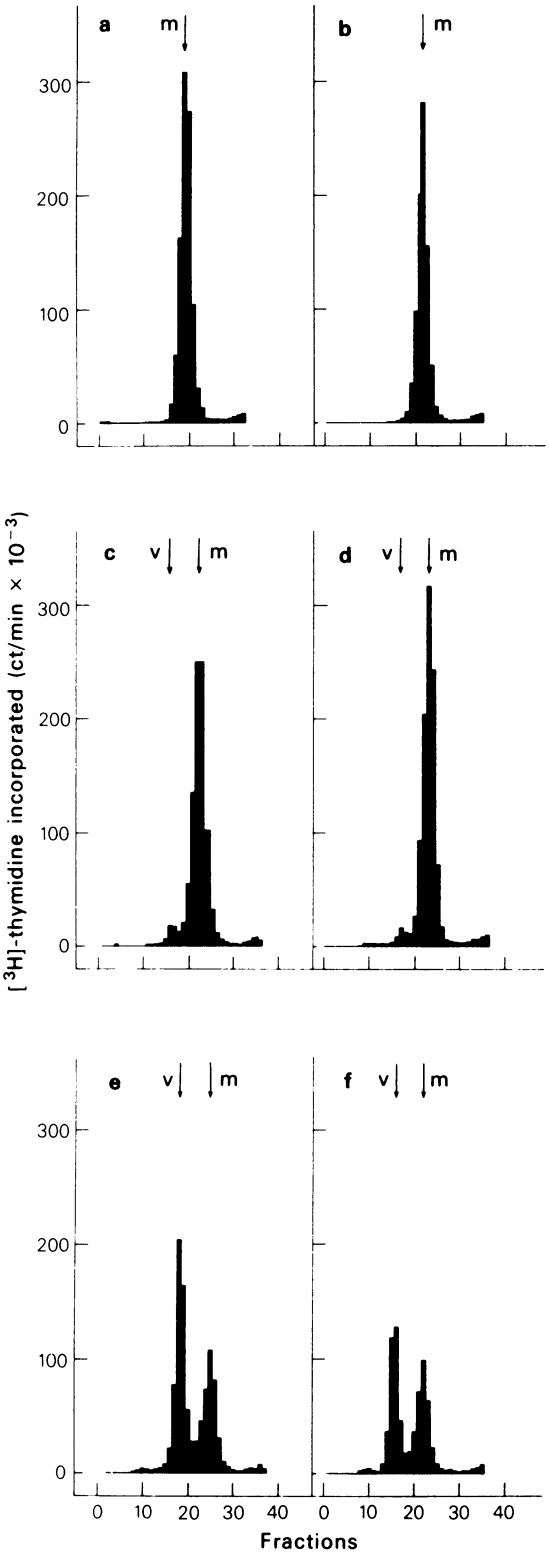
In mice, the oral LD_{50} calculated by the method of Litchfield & Wilcoxon (1949) was 6000 mg/kg and the intraperitoneal LD_{50} was 67 ± 4.5 mg/kg, indicating low enteral absorption of TX 3047. No toxic effect was observed in mice or rats during the oral administration of TX 3047 for 52 days (at 2 and 6 mg/kg daily) as revealed by haematological and pathological examination. In spite of its steroid-like structure (Figure 1), TX 3047 had neither pseudo-gestagen, nor antigonadotrophic activity, and had no effect on the central nervous system of the mice (J. Paris and R. Thevenot, personal communication).

In a preliminary clinical trial labial herpes was treated by local application of TX 3047 (1% w/w in 10% vol/vol ethanol) twice a day. In six patients (Group I) treatment was started within 24 h of the appearance of the very first vesicles, in five patients (Group II) within 3 days and in seven patients (Group III) later than 3 days. All patients believed that their previous untreated herpes lasted an average of 10 days. A reduction of the lesion was observed in all the treated cases. The average duration was reduced by 7 days in Group 1, 4 days in Group II, and 2 days in Group III. TX 3047 did not aggravate the disease and

Table 2 Viral and cellular nucleic acids synthesis in the presence of TX 3047

		Inhibition induced by TX 3047 (150 μg)			*Amount of specific HSV-RNA (%/total RNA)*
		Cellular DNA	*HSV-DNA*	*Cellular RNA*	
Early label	Uninfected cells	57%	–	43%	0
	Infected cells	43%	62%	–	0.46%
	Pretreated cells	0%	0%	–	0.13%
Intermediate label	Uninfected cells	65%	–	53%	0
	Infected cells	39%	62%	–	1.45%
	Pretreated cells	0%	0%	–	0.25%

See text for experimental design. Percentage inhibition was calculated from the radioactivity associated with viral and cellular DNA (or RNA) synthesized in the absence and in the presence of TX 3047.



no pruritis or erythema were observed (Dr P. Cassuto, personal communication).

The limitation of labial herpes in patients could be due to the inactivation of released HSV particles, thus preventing infection of the surrounding cells. A study of the HSV-content in vesicles of treated lesions, determining whether the released virus is immediately inactivated, would help in understanding the mechanism of the spreading of the lesions.

It is interesting that Coryza virus (a rhinovirus) is not sensitive to TX 3047 under the conditions used in this study (Dr H. Metianu, personal communication).

TX 3047 (registered under no 76/17 320) was a kind gift from Theramex Laboratory (Monaco). Thanks are due to T.B. Rogers and M. Center for reviewing the manuscript.

I am indebted to J. Paris and R. Thevenot for communicating their unpublished results concerning the pharmacological properties of TX 3047, and to Dr Cassuto for providing preliminary clinical observations.

Figure 3 Separation of viral and cellular DNA synthesized in the presence of TX 3047. Five ml CsCl gradients were loaded with labelled DNA (see text) centrifuged at 135,000 *g* for 72 h in 50 Ti rotor (Beckman); 10 drop fractions were collected from the bottom of the tubes and labelled DNA counted after TCA precipitation. Arrows indicate the relative position of viral (v) and cellular (m) DNA ($\rho = 1.725$ g/cm^3 and $\rho = 1.700$ g/cm^3, respectively). The distribution of labelled DNA is shown from (a) uninfected cells (cellular DNA); (b) uninfected cells grown in the presence of 100 μg TX 3047; (c) infected cells (early label); (d) infected cells (early label) in the presence of TX 3047; (e) infected cells (late label); (f) infected cells (late label) in the presence of TX 3047.

References

BEN-PORAT, T., & KAPLAN, A.S. (1973). In *The Herpes Viruses*, ed. Kaplan, A.S. pp. 163–220, New York and London: Academic Press.

BEN-ZEEV, A., & BECKER, Y. (1977). Requirement of host cell RNA polymerase II in the replication of Herpes simplex virus in α-ammanitin-sensitive and resistant cell lines. *Virology*, **76**, 246–253.

BRAMHALL, S., NOACK, N., WV, M., & LOEWENBERG, J.R. (1969). A simple colorimetric method for determination of protein. *Ann. Biochem.*, **31**, 146–148.

GAUDRAY, P. (1976). Purification et caracterisation partielles de l'antigene tumoral du virus polyome. *These de doctorat de 3e cycle, Université de Nice.*

HALLIBURTON, I.W., & TIMBURY, M. (1976). Temperature sensitive mutants of Herpes simplex virus type 2: description of three new complementation groups and studies of the inhibition of host cell DNA synthesis. *J. gen. Virol.*, **30**, 207–221.

JAMIESON, A.T., MACNAB, J.C.M., PERBAL, B., & CLEMENTS, J.B. (1976). Virus specified enzyme activity and RNA species in Herpes simplex virus type 1 transformed mouse cells. *J. gen. Virol.*, **32**, 493–508.

JAWETZ, E. (1973). In *The Herpes Viruses*, ed. Kaplan, A.S. pp. 655–688, New York and London: Academic Press.

LITCHFIELD, W.T., & WILCOXON, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.*, **96**, 99–113.

PRUSOFF, W.H., & GOZ, B. (1973). In *The Herpes Viruses* ed. Kaplan, A.S. pp. 641–663, New York and London: Academic Press.

SEIF, R., & CUZIN, F. (1977). Temperature sensitive growth regulation in one type of transformed rat cells by the *tsa* mutant of Polyoma virus. *J. Virol.*, **24**, 721–728.

(Received October 2, 1979. Revised August 19, 1980.)